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(54) Title: ENDOTHELIAL NITRIC OXIDE SYNTHASE

(57) Abstract

A substantially pure preparation of a nucleic acid including a sequence encoding endothelial nitric oxide synthase.

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ENDOTHELIAL NITRIC OXIDE SYNTHASE

Background of the Invention

The observation that acetylcholine-induced vasorelaxation is dependent upon the presence of endothelium led to the discovery of endothelium-derived relaxing factor (EDRF) and, eventually, to its identification as a form of nitric oxide (NO). Furchgott et al., Nature 288, 373-376 (1980); Ignarro et al., G. Proc. Natl. Acad. Sci. U.S.A. 84, 9265-9269 (1987); Palmer et al., Nature 327, 524-526 (1987); Furchgott, R. F. Mechanisms of Vasodilation. New York, Raven Press, 401-414 (1988); Myers et al., Nature 345, 161-163 (1990). NO is synthesized in endothelial cells from L-arginine by nitric oxide synthase (NOS) and diffuses into subjacent smooth muscle cells where it stimulates guanylate cyclase and induces vasodilatation.

Summary of the Invention

In general, the invention features a substantially pure preparation of a nucleic acid sequence which encodes endothelial cell nitric oxide synthase (ECNOS), preferably human ECNOS. In preferred embodiments: the ECNOS nucleic acid sequence is essentially identical to Seq ID No: 1; and the ECNOS nucleic acid sequence encodes an amino acid sequence essentially the same as the amino acid sequence given in Seq ID No: 2.

Preferred embodiments of the invention include: a vector which includes a nucleic acid sequence encoding ECNOS, preferably human ECNOS, e.g., a nucleic acid sequence encoding an amino acid sequence which is essentially that of Seq ID No: 2; a cell which includes the vector; a cell which includes a nucleic acid sequence encoding ECNOS integrated into the genome of the cell (wherein the ECNOS nucleic acid is derived from a different species than the cell); a cell which expresses

the ECNOS encoding nucleic acid; an essentially homogeneous population of cells each of which includes the vector; an essentially homogeneous population of cells each of which includes a sequence encoding ECNOS (wherein the ECNOS nucleic acid is derived from a different organism than the cell) integrated into the genome of the cell.

In another aspect, the invention includes a substantially pure preparation of ECNOS, preferably human ECNOS, e.g., an ECNOS with a sequence essentially identical to Seq ID No: 2.

The invention also includes a therapeutic composition including ECNOS or an enzymatically active fragment thereof, in a pharmaceutically-acceptable carrier. In preferred embodiments the therapeutic composition is essentially free of other proteins of eukaryotic origin.

In another aspect the invention features a method for manufacture of ECNOS including: providing a cell which includes a cloned sequence encoding ECNOS, preferably human ECNOS; culturing the cell in a medium so as to express the sequence; and purifying ECNOS from the cell or the medium. The invention also includes a preparation of ECNOS made by this process.

In another aspect the invention features a purified preparation of an antibody preferably a monoclonal antibody, which specifically binds ECNOS, and an affinity column including the antibody.

In another aspect, the invention features a method of catalyzing the formation of nitric oxide including contacting a substrate, e.g., a guanidino nitrogen, e.g., the terminal guanidino nitrogen of L-arginine, with a substantially purified preparation of ECNOS.

In another aspect, the invention features a method of treating a mammal e.g., a human, having vascular or

circulatory disorder, e.g., systemic or pulmonary hypertension, accelerated-atherosclerosis associated with angioplasty, or coronary artery spasm (Prinzmetal's angina), including administering to the mammal an effective amount of ECNOS.

In another aspect, the invention features a method of determining whether a mammal, e.g., a human, is at risk for a circulatory disorder, e.g., systemic or pulmonary hypertension, accelerated-atherosclerosis associated with angioplasty, or coronary artery spasm (Prinzmetal's angina), including determining the structure of the mammal's ECNOS gene. This can be done by determining the nucleic acid sequence of all or part of the mammal's ECNOS gene, or by restriction fragment length polymorphism analysis. A lesion, e.g., a chromosomal rearrangement, e.g., a deletion, in the mammal's ECNOS gene being predictive of risk for the disorder.

In another aspect, the invention features a method determining whether a mammal, e.g., a human, is at risk for a circulatory disorder, e.g., systemic or pulmonary hypertension, accelerated-atherosclerosis associated with angioplasty, or coronary artery spasm (Prinzmetal's angina), including determining the level and or pattern of expression of the mammal's ECNOS gene, lower than wild type expression or an altered temporal or spatial pattern of expression being indicative of risk for the disorder. In preferred embodiments the level of expression includes measuring the level of ECNOS mRNA in a sample taken from the mammal.

In another aspect, the invention features a method of relaxing a smooth muscle in a mammal, e.g., a human, including administering to the mammal an effective amount of ECNOS.

In another aspect, the invention features a method of increasing the level of endothelial nitric oxide synthase in mammal, e.g., a human, including introducing a nucleic acid sequence encoding endothelial nitric oxide synthase into the mammal and expressing the sequence.

In another aspect, the invention features a method of activating guanylyl cyclase including contacting guanylyl cyclase with a substantially pure preparation of ECNOS.

In another aspect, the invention features a method of inhibiting or reversing platelet aggregation in a mammal, e.g., a human, including administering to the mammal a platelet inhibiting amount of ENOS.

In another aspect, the invention features a method of inhibiting platelet aggregation in a sample comprising contacting the sample with a platelet inhibiting amount of the preparation of ECNOS.

In another aspect, the invention features a method of evaluating a compound, e.g., for anti-inflammatory activities, including contacting the compound with a preparation of ECNOS and determining the ability of the compound to inhibit the production of NO by the preparation. In preferred embodiments the determination is made by monitoring any of the ability of the preparation to change the aggregation properties of platelets, or the ability of the preparation to induce vasodilation.

In another aspect, the invention features a method of evaluating a compound for the ability to inhibit ECNOS including contacting the compound with a preparation of ECNOS and determining the ability of the compound to

inhibit the production of NO by the preparation. In preferred embodiments the determination is made by monitoring any of the ability to produce NO, the ability of the preparation to change the aggregation properties of platelets, or the ability of the preparation to induce vasodilation.

In another aspect, the invention features a method of evaluating a compound for the ability to stimulate ECNOS activity including contacting the compound with a preparation of ECNOS and determining the ability of the compound to promote the production of NO by the preparation. In preferred embodiments the determination is made by monitoring any of the ability to produce NO, the ability of the preparation to change the aggregation properties of platelets, or the ability of the preparation to induce vasodilation. Compounds which stimulate NO production can be used, e.g., to lower blood pressure.

In another aspect, the invention features a method of evaluating a compound for ECNOS-agonist activity including determining the ability of the compound to promote the production of NO, e.g., by contacting the compound with a sample containing ECNOS. In preferred embodiments the determination is made by monitoring any of the ability to produce NO, the ability of the preparation to change the aggregation properties of platelets, or the ability of the preparation to induce vasodilation.

In another aspect, the invention features a method of inhibiting smooth muscle cell proliferation in a mammal, e.g., a human, including administering an effective amount of ENOS to the mammal.

In another aspect, the invention includes, a method of inhibiting smooth muscle cell proliferation after angioplasty in a mammal, e.g., a human, including

performing angioplasty on the mammal, and

administering to the mammal an effective amount of ENOS. The ENOS can be administered before, during, or after angioplasty.

In another aspect the invention features a method of evaluating a treatment, e.g., the administration of a compound, e.g., the administration an arginine analog, for the ability to specifically or preferentially inhibit a first isotype of nitric oxide synthase, e.g., macrophage inducible NO synthase, without substantially inhibiting a second isotype of nitric oxide synthase, e.g., endothelial NO synthase, including contacting the compound with the first isotype and determining if the first isotype is inhibited and contacting the second isotype and determining if the second isotype is substantially inhibited.

The term "substantially pure" describes a preparation which is at least 60% by weight the compound of interest, e.g., a protein, or a polypeptide, e.g., hECNOS. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate method, e.g., in the case of polypeptides by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

The term "substantially pure nucleic acid" refers to preparation of RNA or DNA molecules, e.g., genomic DNA, cDNA, or episomal DNA. With regard to a fragment of a larger nucleic acid, e.g., genomic fragments, fragments of cDNAs, fragments of RNAs, fragments of an episome or plasmid, "substantially pure nucleic acid" refers to a nucleic acid sequence that is not associated with the sequences that flank it in a naturally occurring state, e.g., a DNA fragment that has been removed from the sequences that are adjacent to the fragment, e.g., the

sequences adjacent to the fragment in its normal site in the genome. With regard to essentially unfragmented nucleic acid molecules, e.g., RNA molecules, e.g., mRNA or tRNA molecules, episomal molecules, organellar DNA, e.g., chloroplast or mitochondrial DNA, or essentially whole genomic molecules, e.g., viral genomes, or chromosomes, "substantially pure nucleic acid" refers to a nucleic acid preparation that is less than 50% by weight other components, e.g., proteins, lipids, or other nucleic acids, that naturally accompany the nucleic acid. With regard to synthetic nucleic acid sequences "substantially pure nucleic acid" refers to a preparation in which at least 50% by weight of the nucleic acid is the nucleic acid of interest.

The term "homologous" refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half, e.g., 5 of 10, of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC'5 and 3'TATGGC'5 share 50% homology. By "substantially homologous" is meant largely but not wholly homologous.

The invention is useful for: investigating the role of ECNOS in endothelial regulation of vascular tone; investigating the molecular basis of a variety of vascular disorders, e.g., systemic or pulmonary

hypertension, accelerated-atherosclerosis associated with angioplasty, or coronary artery spasm (Prinzmetal's angina); inducing the relaxation of smooth muscle; supplying a transgenic source of ECNOS to a mammal, e.g., a mammal with a vascular disorder or a mutant ECNOS locus; screening for inhibitors of ECNOS, for use, e.g., as anti-inflammatory agents; determining if a mammal is at risk for a vascular disorder, e.g., systemic or pulmonary hypertension, accelerated-atherosclerosis associated with angioplasty, or coronary artery spasm (Prinzmetal's angina) by determining the structure or level of expression of the ECNOS gene in the mammal; generating antibodies, preferably monoclonal antibodies, useful in studying the function and distribution of ENOS; and discovering stimulators, agonists, and inhibitors of ENOS, (stimulators and agonists are useful for decreasing blood pressure, inhibitors are useful for increasing blood pressure).

Other features and advantages of the invention will be apparent from the following description and from the claims.

Detailed Description

The drawings are first briefly described.

Drawings

Fig. 1 is the sequence of hECNOS (Seq ID No: 1).

Fig. 2 is an alignment of the amino acid sequences of ECNOS (Seq ID No: 2) with brain NOS (Seq ID No: 3).

Fig. 3 is a RNA blot hybridization showing ECNOS expression in spleen, kidney, lung, and HUVEC.

Fig. 4 is a photograph showing the localization of NADPH diaphorase activity in bovine pulmonary artery endothelial cells (panel A), in NIH/3T3 cells expressing the human endothelial NOS cDNA (panel B), and in untransfected NIH/3T3 cells (panel C).

Fig. 5 is a graph of the catalytic activity of ECNOS in control and transfected cells.

Overview

A human cDNA encoding vascular endothelial nitric oxide synthase which also confers NADPH diaphorase activity on transfected NIH/3T3 cells has been isolated. Endothelial cell-produced NO acts as an endogenous nitrovasodilator by activating soluble guanylyl cyclase, Moncada et al., Pharmacol. Rev. 43, 109-142 (1991), in vascular smooth muscle cells. A similar cGMP-dependent inhibition of platelet aggregation and adhesion accounts for the potent antithrombotic action of NO, Radomski et al., Br. J. Pharmacol. 92, 639-646 (1987). The crucial role of endothelial cell NO production in controlling arterial tone and modulating platelet aggregation and adhesion has only recently been recognized, Marsden et al., Seminars in Nephrology 11, 169-185 (1991), Vane et al., N. Engl. J. Med. 323, 27-36 (1990), and its significance in the pathophysiology of pulmonary and systemic hypertension and atherosclerotic disorders is of great interest.

Cloning Human ECNOS

Fig. 1 shows the sequence of a human ECNOS (Seq ID No: 1). Oligonucleotides corresponding to amino acids in domains shared by cytochrome P-450 reductase and the recently-identified brain NOS, Bredt et al. Nature 351, 714-718 (1991), were synthesized to amplify a partial cDNA encoding a bovine endothelial cell NOS. This partial cDNA was used to isolate a cDNA was used to isolate a cDNA encoding a human vascular endothelial NOS. The translated human protein is 1203 amino acids in length and shares approximately 50% percent of its amino acid sequence with brain NOS.

To identify genes which encode nitric oxide synthases and are expressed in vascular endothelial

cells, degenerate oligonucleotide primers based upon the sequence of a specific domain of brain NOS, Bredt et al. (1991), cDNA prepared from bovine aortic endothelial cell RNA were synthesized. The primer sequences corresponded to the NADPH-ribose and NADPH-adenine cofactor binding sites. These sites are highly conserved between brain NOS and cytochrome P450 reductase Bredt et al., (1991).

Degenerate oligonucleotides corresponding to the NADPH-ribose site, 5'-CGGGATCCGGNACNGGNATHGCNCCNTT-3' (Seq ID No: 4), and complementary to the NADPH-adenine site, 5'-GCGAATTCNCCRCANACRTADATRTG-3' (Seq ID No: 5), were used to amplify NOS-related cDNAs from bovine aortic endothelial cell RNA using the polymerase chain reaction (30 cycles, denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes, and extension at 72°C for 3 minutes). PCR products approximately 350-bp in length were purified by agarose-gel electrophoresis and cloned into pGEM7zf (Promega). The nucleotide sequences of several partial cDNA clones were determined; one of these showed significant amino-acid homology with portions of brain NOS. A restriction fragment prepared from this bovine cDNA was used to screen a λGT10 cDNA library prepared from lipopolysaccharide-stimulated human umbilical vein endothelial cells, Staunton et al., Cell 52, 925-933 (1988). cDNA inserts from a hybridizing bacteriophage were subcloned in pUC19. Nucleotide sequence determination of the longest cDNA was performed by the dideoxynucleotide chain termination method using nested deletions. Both sense and antisense DNA strands were sequenced.

One of the amplified partial cDNAs was approximately 350-bp in length and encoded a peptide which shared significant amino acid homology with segments of brain NO synthase and a λGT10 cDNA library prepared from lipopolysaccharide-stimulated human

umbilical vein endothelial cells, Staunton et al., (1988), was screened with this partial cDNA, and hybridizing bacteriophage were isolated. A clone with a 4099-bp insert was thus obtained. It contained a 3609-bp open reading from encoding a 1203-amino acid protein. The first methionine lies within a Kozak consensus sequence for initiation of translation, Kozak, Cell 44, 283-292 (1986).

At the amino acid level, this endothelial cell protein shares only 52 percent of its amino acid sequence with brain NOS though striking homology is evident at sequences corresponding to flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and NADPH binding regions, see Fig. 2. (Fig. 2 shows the amino acid sequence alignment of endothelial NOS (Seq ID No: 2) with brain NOS (Seq ID No: 3). The amino acid sequence of human endothelial NOS is shown in upper-case single letter code. The sequence of rat brain NOS is shown in lower case. Residue numbers are indicated on the left with the final residue number portrayed on the bottom right. Identical amino acids are indicated by a solid line and gaps introduced to maintain sequence alignment are represented by dots. Endothelial NOS shares 52% of its amino acid sequence with the brain enzyme. Sequences which are highly conserved between the two proteins and which probably represent contact sites for binding of enzyme cofactors, Bredt et al., (1991), are enclosed in boxes, including FMN, FAD-pyrophosphate and-isoalloxazine groups, and NADPH-adenine and-ribose groups.)

This degree of homology may account for the recognition of NOS-immunoreactivity in endothelial cells by an antiserum raised against brain NOS, Bredt et al., (1990). In contrast, the two proteins differ markedly elsewhere, e.g., at their amino and carboxyl termini. The encoded peptide is predicted to be about 133 kDa in

size which is smaller than brain NO synthase (predicted molecular weight 160 kDa) and closer to the 135-kDa type III endothelial cell constitutive NO synthase, recently characterized by Pollock et al., Pollock et al. Proc. Natl. Acad. Sci. U.S.A. 88, 10480-10484 (1991).

In vivo Expression and Tissue Distribution of Human ECNOS

RNA blot hybridization confirmed that the gene encoding this endothelial NO synthase was expressed in human umbilical vein endothelial cells (HUVEC) as well as human lung, kidney, and spleen, see Fig. 3. (Fig. 3 shows the identification of endothelial NOS mRNA by RNA blot hybridization. Expression of the endothelial NOS gene was detected in cultured human umbilical vein endothelial cells and human spleen, lung, and kidney. The mRNA is approximately 4300 nucleotides long. The migration of ribosomal RNA is indicated (28S and 18S)).

RNA was extracted from human tissues and cells using the guanidinium isothiocyanate/cesium chloride method, Sambrook Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York (1989), hereby incorporated by reference, fractionated on a formaldehyde agarose gel (10μg per lane), and transferred to a nylon membrane. Blots were hybridized with the ^{32}P-labeled 1.4-kb BamH1-EcoR1 restriction fragment at 42°C, washed under stringent conditions and subjected to autoradiography for 7 days.

This tissue distribution of expression differs markedly from that of the brain NOS gene, Bredt et al. (1991). The mRNA encoding the human endothelial gene was approximately 4300-nucleotides in length and much smaller than brain NOS mRNA. The presence of abundant mRNA in unstimulated endothelial cells and its homology to the constitutively expressed brain NOS further suggests that the endothelial cell cDNA encodes a constitutively

expressed NOS, Radomski et al., Proc. Natl. Acad. Sci. U.S.A. 87, 10043-10047 (1990).

Expression of the ECNOS cDNA by stable transfection conferred NADPH diaphorase activity upon NIH 3T3 cells.

Recent observations by Hope et al., Hope et al., Proc. Natl. Acad. Sci. U.S.A. 88, 2811-2814 (1991), and Dawson et al., Dawson et al., Proc. Natl. Acad. Sci. U.S.A. 88, 7797-7801 (1991), indicate that brain NOS has NADPH diaphorase activity. To determine whether the NADPH diaphorase histochemical assay might serve as a marker for endothelial NO synthase the endothelial cell cDNA was ligated 3′ to the SV40 promoter in the pSPORT expression vector and transfected into NIH/3T3 cells (described below). Bovine pulmonary artery endothelial cells incubated in the presence of NADPH and nitro blue tetrazolium showed small amounts of blue cytoplasmic staining consistent with low levels of NADPH diaphorase activity, Fig. 4A. Control NIH/3T3 cells contained no detectable blue staining, Fig. 4C. In contrast, transfected cells with high levels of the endothelial cell mRNA (as measured by RNA blot hybridization) showed abundant blue cytoplasmic staining, Fig. 4B. The presence of high levels of NADPH diaphorase activity in transfected cells demonstrated that the expressed cDNA encoded a protein which was functional enzyme with properties similar to brain NOS.

The human endothelial NOS cDNA was cloned into the EcoR1 site of pSPORT (Bethesda Research Laboratories). The expression plasmid together with a plasmid encoding neomycin resistance were transfected into NIH/3T3 cells by the calcium-phosphate method, Sambrook Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York (1989), hereby incorporated by reference. Following selection in the antibiotic G418, resistant clones containing endothelial NOS mRNA were

identified. NADPH diaphorase staining was performed by incubating cells with 1 mH NADPH, 0.2 mM nitro blue tetrazolium, 0.1 M Tris-HCl (pH 7.2), and 0.2% Triton X-100 for 30 min at 37°C, Dawson et al., Proc. Natl. Acad. Sci. U.S.A. 88:7797-7801 (1991). Cells were photographed at the same magnification under bright-field illumination. A blue cytoplasmic staining pattern is indicative of NADPH diaphorase activity.

<u>ECNOS transfected NIH 3T3 release NO</u>

To test whether transfected cells release NO, a co-incubation assay, Marsden et al., Am. J. Physiol. 258:1295-1303 (1990), was used, wherein stimulation of soluble guanylate cyclase in a reporter cell monolayer (rat fetal lung fibroblasts, RFL-6 cells) reflects production of NO. Control and transfected NIH/3T3 cells, plated on glass coverslips, were juxtaposed to RFL-6 cells in tissue culture dishes in the presence of 3-isobutyl-1-methylxanthine for 30 minutes. Cell monolayers were subsequently extracted, and cGMP concentrations were measured by radioimmunoassay. cGMP concentrations did not differ between RFL-6 cells exposed to uncoated coverslips or to coverslips coated with NIH/3T3 cells (data not shown). In contrast, RFL-6 cells exposed to coverslips coated with transfected cells contained significantly increased concentrations of cGMP. To confirm that the increased cGMP production in reporter cells was due to stimulation by NO, transfected cells and RFL-6 cells were co-incubated in the presence of the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), Rees et al., Br. J. Pharmacol, 101, 746-752 (1990). As shown in Fig. 5, the inhibitor significantly decreased the ability of transfected cells to stimulate cGMP production in RFL-6 cells. (Fig. 5 shows the functional expression of endothelial NOS enzyme activity. NOS catalytic activity in transfected 3T3 cells was assessed

by measuring the cGMP response to nitric oxide in a co-incubation assay using reporter rat lung fibroblasts (RFL-6 cells). Cellular cGMP content (pmoles per well) was measured following co-incubation of RFL-6 cells with untransfected NIH/3T3 cells (CONTROL 3T3) and with transfected cells expressing the human endothelial NOS cDNA (hECNOS-TRANSFECTED 3T3) in the absence (UNTREATED) and presence of N^G-nitro-L-arginine methyl ester (L-NAME-TREATED). Data are expressed as means $\pm$ standard error of six determinations from representative experiment.

The co-incubation assay performed for measuring NO production was a modification of the technique described by Marsden et al. (1990). Rat fetal fibroblasts (RFL-6 cells; American Type Culture Collection #CCL191) were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum and glutamine in 12 well tissue culture plates. Control and transfected 3T3 cells were grown on coverslips (approximately 10^5 control cells per coverslip and $6x10^4$ transfected cells per coverslip in the representative experiment shown). RFL-6 cells were pretreated for 10 minutes with 200 U/ml superoxide dismutase and 0.1 mM 3-isobutyl-1-methylxanthine. Coverslips were then juxtaposed to the reporter cell monolayer for 30 min. To inhibit NOS activity, identical wells were treated with 0.5 mM L-NAME for ten minutes prior to addition of coverslips. Cellular cGMP was extracted in 0.1 M HCl, Beasley et al., J. Clin. Invest. 87, 602-608 (1991), and was quantitated by radioimmunoassay after acetylation (Biomedical Technologies Inc.).

Use

The peptides of the invention may be administered to a mammal, particularly a human, in one of the traditional modes (e.g., orally, parenterally, transdermally, or transmucosally), in a sustained release formulation using a biodegradable biocompatible polymer, or by on-site delivery using micelles, gels and liposomes or by transgenic modes.

Other Embodiments

The invention includes any protein which is 55% or more homologous to hECNOS (Seq ID No: 2). Preferably the protein is at least 65% or more, more preferably 80% or more, and most preferably 95% or more, homologous to hECNOS. Also included are: allelic variations; natural mutants; induced mutants. Also included are chimeric polypeptides that include an hECNOS.

The invention also includes analogs of naturally occurring hECNOS. Analogs can differ from naturally occurring hECNOS by amino acid sequence differences or by modifications that do not affect sequence, or by both. Analogs of the invention will generally exhibit at least 70%, more preferably 80%, more preferably 90%, and most preferably 95% or even 99%, homology with all or part of a naturally occurring hECNOS sequence. The length of comparison sequences will generally be at least about 8 amino acid residues, usually at least 20 amino acid residues, more usually at least 24 amino acid residues, typically at least 28 amino acid residues, and preferably more than 35 amino acid residues. Modifications include *in vivo*, or *in vitro* chemical derivatization of polypeptides, e.g., acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps, e.g., by exposing the

polypeptide to enzymes that affect glycosylation derived from cells that normally provide such processing, e.g., mammalian glycosylation enzymes. Also embraced are versions of the same primary amino acid sequence that have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine. Analogs can differ from naturally occurring hECNOS by alterations of their primary sequence. These include genetic variants, both natural and induced. Induced mutants may be derived by various techniques, including random mutagenesis of the encoding nucleic acids using irradiation or exposure to ethanemethylsulfate (EMS), or may incorporate changes produced by site-specific mutagenesis or other techniques of molecular biology. See, Sambrook, Fritsch and Maniatis (1989), Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, hereby incorporated by reference. Also included are analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids.

In addition to substantially full-length polypeptides, the invention also includes biologically active fragments of the polypeptides. As used herein, the term "fragment", as applied to a polypeptide, will ordinarily be at least about 10 contiguous amino acids, typically at least about 20 contiguous amino acids, more typically at least about 30 contiguous amino acids, usually at least about 40 contiguous amino acids, preferably at least about 50 contiguous amino acids, and most preferably at least about 60 to 80 or more contiguous amino acids in length. Fragments of hECNOS can be generated by methods known to those skilled in the art. The ability of a candidate fragment to exhibit a biological activity of hECNOS can be assessed by methods

known to those skilled in the art. Also included are hECNOS polypeptides containing amino acids that are normally removed during protein processing, including additional amino acids that are not required for the biological activity of the polypeptide, or including additional amino acids that result from alternative mRNA splicing or alternative protein processing events.

A hECNOS polypeptide, fragment, or analog is biologically active if it exhibits a biological activity of a naturally occurring hECNOS, e.g., the ability to catalyze the synthesis of NO from L-arginine.

Nucleic acids and proteins of the invention can be used to screen treatments, e.g., the administration of compounds, e.g., the administration of an arginine analog, for isotype-selective NO synthase inhibition. There are numerous isotypes of NO synthase in the body and it can be desirable to inhibit one isotype but not another. For example, macrophage inducible NO synthase is active in sepsis. It would be desirable to inhibit inducible macrophage NO synthase in sepsis patients (and thus increase their blood pressure) without substantially inhibiting endothelial NO synthase. A treatment, e.g., a compound, can be screened for the ability to specifically or preferentially inhibit a first isotype of NO synthase without inhibiting a second isotype of NO synthase.

A treatment, e.g., the administration of a compound, specifically or preferentially inhibits a first isotype if, at a predetermined concentration or dose, it results in a substantial effect on a parameter determined by the activity of the first isotype, e.g., macrophage NO synthase dependent changes in blood pressure in sepsis patients, but does not result in a substantial effect on a parameter determined by the action of the second isotype, e.g., endothelial NO synthase dependent changes in the vascular system. Preferrably, the treatment

results in a greater decrease in NO production of the first isotype than in NO production by the second isotype.

Other embodiments are within the following claims.

What is claimed is:

Claims

1. A substantially pure preparation of a nucleic acid comprising a sequence encoding endothelial nitric oxide synthase.

2. The substantially pure preparation of a nucleic acid of claim 1, wherein said sequence is essentially identical to Seq ID No: 1.

3. The substantially pure preparation of a nucleic acid of claim 1, wherein said sequence encodes a product which comprises essentially the amino acid sequence given in Seq ID No: 2.

4. A vector comprising the nucleic acid of claim 1.

5. A cell comprising the nucleic acid of claim 4.

6. The cell of claim 5, wherein said cell expresses a polypeptide encoded by said nucleic acid.

7. An essentially homogeneous population of cells, each of which comprises the nucleic acid of claim 1.

8. A substantially pure preparation of endothelial nitric oxide synthase.

9. A therapeutic composition comprising endothelial nitric oxide synthase or an enzymatically active fragment thereof, in a pharmaceutically-acceptable carrier.

10. The preparation of claim 9, said preparation being essentially free of other proteins of eukaryotic origin.

11. A method for the manufacture of endothelial nitric oxide synthase comprising:

providing the cell of claim 5;

culturing said cell in a medium so as to express said sequence; and

purifying endothelial nitric oxide synthase from said cell or said medium.

12. The preparation of claim 11, wherein said endothelial nitric oxide synthase comprises an amino acid sequence essentially identical to that of Seq ID No: 2.

13. A preparation of endothelial nitric oxide synthase made by the process of claim 11.

14. A method of catalyzing the formation of nitric oxide comprising contacting L-arginine with a substantially purified preparation of endothelial nitric oxide synthase.

15. A method of treating a mammal having hypertension comprising administering to said mammal a blood pressure lowering amount of endothelial nitric oxide synthase.

16. A method of determining whether a mammal is at risk for a circulatory disorder comprising

determining the nucleic acid sequence of the mammals ECNOS gene, a lesion in the mammals ECNOS gene being predictive of risk for said disorder.

17. The method of claim 16, wherein said lesion is a deletion.

18. A method determining whether the mammal is at risk for a circulatory disorder comprising

determining the level of expression of the mammals ECNOS gene, lower than wild type expression being indicative of risk for said disorder.

19. The method of claim 18 wherein said determination the level of expression comprises measuring the level of endothelial nitric oxide synthase RNA in a sample taken from said mammal.

20. A method of relaxing a smooth muscle in a mammal comprising administering to said mammal an effective amount of endothelial nitric oxide synthase.

21. A method of increasing the level of endothelial nitric oxide synthase in a mammal comprising introducing a nucleic acid sequence encoding endothelial nitric oxide synthase into said mammal and expressing said sequence.

22. A method of activating guanylyl cyclase comprising contacting guanylyl cyclase with the preparation of claim 8.

23. A method of inhibiting platelet aggregation in a mammal comprising administering to said mammal a platelet inhibiting amount of the preparation of claim 8.

24. A method of inhibiting platelet aggregation in a sample comprising contacting said sample with a platelet inhibiting amount of the preparation of claim 8.

25. A method of evaluating a compound comprising contacting said compound with the preparation of claim 8 and determining the ability of said compound to inhibit the production of NO by said preparation.

26. A method of inhibiting smooth muscle cell proliferation in a mammal comprising administering an effective amount of endothelial nitric oxide synthase to said mammal.

27. A method of inhibiting smooth muscle cell proliferation after angioplasty in a mammal comprising

performing angioplasty on said mammal, and

administering to said mammal an effective amount of endothelial nitric oxide synthase.

28. A method of evaluating a treatment for the ability to preferentially inhibit a first isotype of nitric oxide synthase without substantially inhibiting a second isotype of nitric oxide synthase comprising contacting said compound with said first isotype and determining if said isotype is inhibited and contacting said second isotype and determining if said second isotype is substantially inhibited.

FIG. 1

SEQ ID NO: 1
The nucleotide sequence of hECNOS:

```
1    gaattcccac tctgctgcct gctccagcag acggacgcac agtaacatgg
51   gcaacttgaa gagcgtggcc caggagcctg ggccaccctg cggcctgggg
101  ctggggctgg gccttgggct gtgcggcaag cagggcccag ccaccccggc
151  ccctgagccc agccgggccc cagcatccct actcccacca gcgccagaac
201  acagcccccc gagctccccg ctaacccagc cccagaggg gcccaagttc
251  cctcgtgtga agaactggga ggtggggagc atcacctatg acaccctcag
301  cgcccaggcg cagcaggatg ggccctgcac cccaagacgc tgcctgggct
351  ccctggtatt tccacggaaa ctacagggcc ggccctcccc cggccccccg
401  gcccctgagc agctgctgag tcaggcccgg gacttcatca accagtacta
451  cagctccatt aagaggagcg gctcccaggc ccacgaacag cggcttcaag
501  aggtggaagc cgaggtggca gccacaggca cctaccagct tagggagagc
551  gagctggtgt tcggggctaa gcaggcctgg cgcaacgctc cccgctgcgt
601  gggccggatc cagtggggga agctgcaggt gttcgatgcc cgggactgca
651  ggtctgcaca ggaaatgttc acctacatct gcaaccacat caagtatgcc
701  accaaccggg gcaaccttcg ctcggccatc acagtgttcc cgcagcgctg
751  ccctggccga ggagacttcc gaatctggaa cagccagctg gtgcgctacg
801  cgggctaccg gcagcaggac ggctctgtgc gggggaccc agccaacgtg
851  gagatcaccg agctctgcat tcagcacggc tggaccccag gaaacggtcg
901  cttcgacgtg ctgcccctgc tgctgcaggc cccagatgag cccccagaac
951  tcttccttct gccccccgag ctggtccttg aggtgcccct ggagcacccc
1001 acgctggagt ggtttgcagc cctgggcctg cgctggtacg ccctcccggc
1051 agtgtccaac atgctgctgg aaattggggg cctggagttc cccgcagccc
1101 ccttcagtgg ctggtacatg agcactgaga tcggcacgag gaacctgtgt
1151 gaccctcacc gctacaacat cctggaggat gtggctgtct gcatggacct
1201 ggatacccgg accacctcgt ccctgtggaa agacaaggca gcagtggaaa
```

FIG. 1 (cont.)

1251 tcaacgtggc cgtgctgcac agttaccagc tagccaaagt caccatcgtg
1301 gaccaccacg ccgccacggc ctctttcatg aagcacctgg agaatgagca
1351 gaaggccagg gggggctgcc ctgcagactg ggcctggatc gtgcccccca
1401 tctcgggcag cctcactcct gttttccatc aggagatggt caactatttc
1451 ctgtccccgg ccttccgcta ccagccagac ccctggaagg ggagtgccgc
1501 caagggcacc ggcatcacca ggaagaagac ctttaaagaa gtggccaacg
1551 ccgtgaagat ctccgcctcg ctcatgggca cggtgatggc gaagcgagtg
1601 aaggcgacaa tcctgtatgg ctccgagacc ggccgggccc agagctacgc
1651 acagcagctg gggagactct tccggaaggc ttttgatccc cgggtcctgt
1701 gtatggatga gtatgacgtg gtgtccctcg aacacgagac gctgGTgctg
1751 gtggtaacca gcacatttgg gaatggggat cccccggaga atggagagag
1801 ctttgcagct gccctgatgg agatgtccgg cccctacaac agctcccctc
1851 ggccggaaca gcacaagagt tataagatcc gcttcaacag catctcctgc
1901 tcagacccac tggtgtcctc ttggcggcgg aagaggaagg agtccagtaa
1951 cacagacagt gcaggggccc tgggcaccct caggttctgt gtgttcgggc
2001 tcggctcccg ggcatacccc cacttctgcg cctttgctcg tgccgtggac
2051 acacggctgg aggaactggg cggggagcgg ctgctgcagc tgggccaggg
2101 cgacgagctg tgcggccagg aggaggcctt ccgaggctgg gcccaggctg
2151 ccttccaggc cgcctgtgag accttctgtg tgggagagga tgccaaggcc
2201 gccgcccgag acatcttcag ccccaaacgg agctggaagc gccagaggta
2251 ccggctgagc gcccaggccg agggcctgca gttgctgcca ggtctgatcc
2301 acgtgcacag gcggaagatg ttccaggcta caatccgctc agtggaaaac
2351 ctgcaaagca gcaagtccac gagggccacc atcctggtgc gcctggacac
2401 cggaggccag gaggggctgc agtaccagcc gggggaccac ataggtgtct
2451 gcccgcccaa ccggcccggc cttgtggagg cgctgctgag ccgcgtggag
2501 gacccgccgg cgcccactga gcccgtggca gtagagcagc tggagaaggg
2551 cagccctggt ggccctcccc ccggctgggt gcgggacccc cggctgcccc

FIG. 1 (cont.)

2601 cgtgcacgct gcgccaggct ctcaccttct tcctggacat cacctcccca
2651 cccagccctc agctcttgcg gctgctcagc accttggcag aagagcccag
2701 ggaacagcag gagctggagg ccctcagcca ggatccccga cgctacgagg
2751 agtggaagtg gttccgctgc cccacgctgc tggaggtgct ggagcagttc
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2851 ccagccccgg tactactcag tcagctcggc acccagcacc cacccaggag
2901 agatccacct cactgtagct gtgctggcat acaggactca ggatgggctg
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3151 agggctgcag cccactccca tgactttggt gttcggctgc cgatgctccc
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3351 gcgtgctgtg cctcgagcgg ggccacatgt ttgtctgcgg cgatgttacc
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3451 cgacatggag ctggacgagg ccggcgacgt catcggcgtg ctgcgggatc
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3551 gtgacaagcc gcatacgcac ccagagcttt tccttgcagg agcgtcagtt
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3901 agggcctact gccacccgct tcctgtttct tagtccgaat gttagattcc
3951 tcttgcctct ctcaggagta tcttacctgt aaagtctaat ctctaaatca
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4051 gaactactaa agtgcctacc ccagctcaaa aaaaaaaaaa aaaaaaaaa

```
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233  lqvdrldqtahkappiqqandrvfndlwqkdnvpvilnnpyseteqspt
51   EMSPPSSPLTQPPEGPKFPRVKNWEVGSITYDTLSAQAQQDGPCTPRRCL
283  sgtqspcknqspercprfltvknwetevvltdtlhltsqlstqcteniwm
101  GSLVFPRKLQGRPSPGPPAPEQLLSQARDFINQYYSSIKRSGSQAHEQRL
333  qsiqlp.sqhtrkpedvrtkdqlfplaksfldqyysslkrfgskahmdrl
151  QEVEAEVAATGTYQLRESELVFGAKQAWRNAPRCVGRIQWGKLQVFDARD
382  eevnkeiestetyqlkdtellygakhawrnasrcvgriqwsklqvfdard
201  CRSAQEMFTYICNHIKYATNRGNLRSAITVFPQRCPGRGDFRIWNSQLVR
432  cttahgmfnyicnhvkyatnkgnirsaitifpqrtdgkhdfrvwnsqlir
251  YAGYRQQDGSVRGDPANVEITELCIQHGWTPGNGRFDVLPLLLQAPDEPP
482  yagykqpdgstlgdpanvqfteiciqqgwkaprgrfdvlpllqangndp
301  ELFLLPPELVLEVPLEHPTLEWFAALGLRWYALPAVSNMLLEIGGLEFPA
532  elfqippelvlevpirhpkfdwfkdlglkwyglpavsnmlleiggleface
351  APFSGWYMSTEIGTRNLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAV
582  cpfsgwymgteigvrdycdnsrynileevakkmdldmrktsslwkdqalv
401  EINVAVLHSYQLAKVTIVDHHAATASFMKHLENEQKARGGCPADWAWIVP
632  einiavlysfqsdkvtivdhhsatesfikhmeneyrcrggcpadwvwivp
451  PISGSLTPVFHQEMVNYFLSPAFRYQPDPWKGSAARGTGIT....RKKTF
682  pmsgsitpvfhqemlnyrltpsfeyqpdpwnthvwkgtngtptkrraigf
497  KEVANAVKISASLMGTVMAKRVKATILYGSETGRAQSYAQQLGRLFRKAF
732  kklaeavkfsaklmgqamakrvkatilyatetgksqayaktlceifkhaf
547  DPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAAALMEMSGP
782  dakamsmeeydivhlehealvlvvtstfgngdppengekfgcalmemrhp
597  YNSSPRPEQHKSYKIRFNSISCSDPLVSSWRRKRKESSNTDSAGALGTLR
832  ...nsvqeerksykvrfnsvssysdsrkssgdgpdlrdnfestgplanvr
647  FCVFGLGSRAYPHFCAFARAVDTRLEELGGERLLQLGQGDELCGQEEAFR
879  fsvfglgsrayphfcafghavdtlleelggerilkmreqdelcgqeeafr
697  GWAQAAFQAACETFCVGED..AKAAARDIFSPKRSWKRQRYRLSAQAEGL
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745  QLLPGLIHVHRRKMFQATIRSVENLQSSKSTRATILVRLDTGGQEGLQYQ
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795  PGDHIGVCPPNRPGLVEALLSRVEDPPAPTEPVAVEQL.EKGSPGGPPPG
1029 pgdhlgvfpgnhedlvnallerledappanhvvkvemleerntalgvism
844  WVRDPRLPPCTLRQALTFFLDITSPPSPQLLRLLSTLAEEPREQQELEAL
1079 wkdesrlppctifqafkyyldittpptplqlqqfaslatnekekqrllvl
894  SQDPRRYEEWKWFRCPTLLEVLEQFPSVALPAPLLLTQLPLLQPRYYSVS
1129 skglqeyeewkwgknptmvevleefpsiqmpatllltqlsllqprysis
944  SAPSTHPGEIHLTVAVLAYRTQDGLGPLHYGVCSTWLSQLKPGDPVPCFI
1179 sspdmypdevhltvaivsyhtrdgegpvhhgvcsswlnriqaddvvpcfv
994  RGAPSFRLPPDPSLPCILVGPGTGIAPFRGFWQERLHDIESKGLQPTPMT
1229 rgapsfhlprnpqvpcilvgpgtgiapfrsfwqqrqfdiqhkgmnpcpmv
1043 LVFGCRCSQLDHLYRDEVQNAQQRGVFGRVLTAFSREPDNPKTYVQDILR
1279 lvfgcrqskidhiyreetlqaknkgvfrelytaysrepdrpkkyvqdvlq
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1329 eqlaesvyralkeqgghiyvcgdvtmaadvlkaiqrimtqqgklseedaq
1143 DVIGVLRDQQRYHEDIFGLTLRTQEVTSRIRTQSFSLQERQLRGAVPWAF
1379 vfisrlrddnrybedifgvtlrtyevtnrlrsesiafleesktdadevfss 1429
```

FMN

FAD-pyrophosphate

FAD-isoalloxazine

NADPH-ribose

NADPH-adenine

Seq ID No: 3

1194 DPPGSDTRSP

Seq ID No: 2

Fig. 3

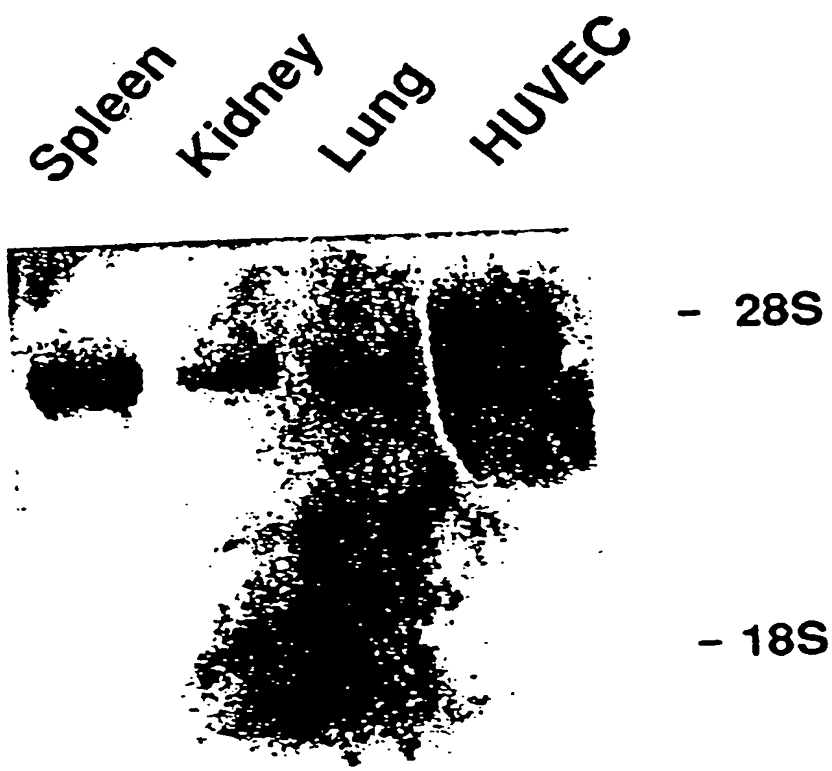


Fig. 4

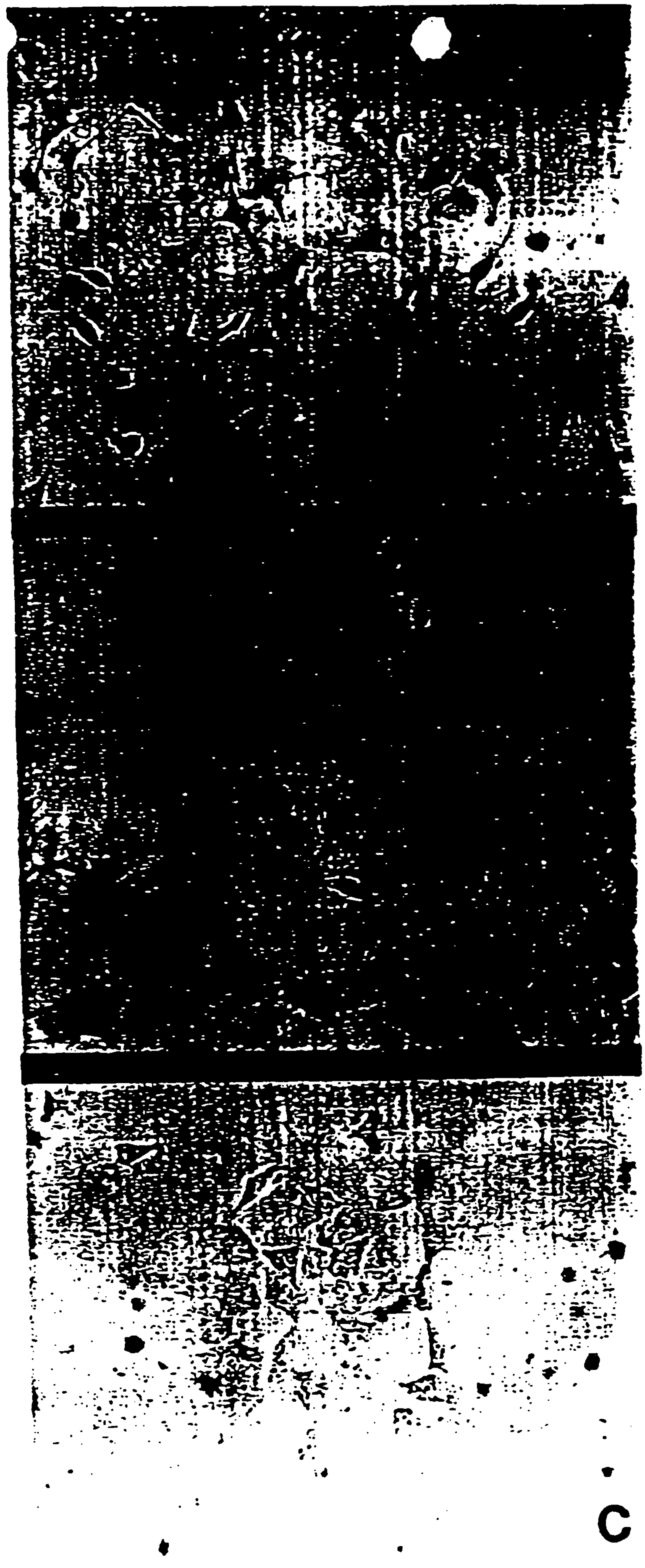
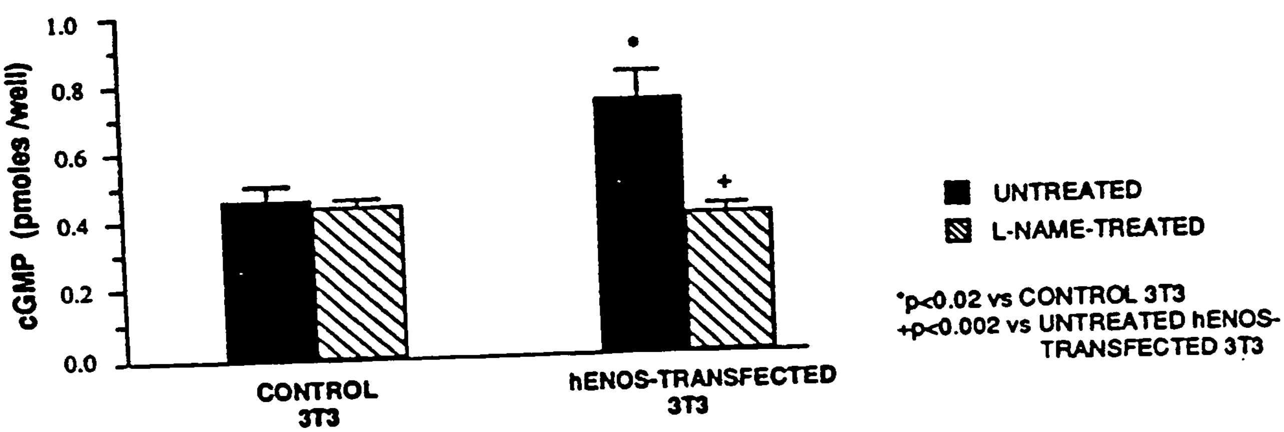


Fig. 5


cGMP (pmoles /well)
1.0
0.8
0.6
0.4
0.2
0.0
CONTROL 3T3
hENOS-TRANSFECTED 3T3
UNTREATED
L-NAME-TREATED
*p<0.02 vs CONTROL 3T3
+p<0.002 vs UNTREATED hENOS-TRANSFECTED 3T3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01951

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 320.1, 252.3, 191, 168, 6, 232, 25; 536/23.2; 424/94.4; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, BIOSIS, EMBASE, LIFESCI, APS
search terms: nitric oxide synthase or synthetase, endothel?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 267, No. 21, issued 25 July 1992, S.P. Janssens et al., "Cloning and Expression of a cDNA Encoding Human Endothelium-derived Relaxing Factor/Nitric Oxide Synthase", pages 14519-14522, entire document.	1-8, 11-14 9-10, 15-28
P,X Y	FEBS LETTERS, Volume 307, No. 3, issued August 1992, P.A. Marsden et al., "Molecular Cloning and Characterization of Human endothelial nitric oxide Synthase", pages 287-293, entire document.	1-7 8-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
04 June 1993

Date of mailing of the international search report
14 JUN 1993

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, volume 89, issued July 1992, S. Lamas et al., "Endothelial Nitric Oxide Synthase: Molecular Cloning and Characterization of a distinct constitutive Enzyme Isoform", pages 6348-6352, entire document.	1, 4-8, 11, 13, 14 2-3, 9-10, 15-28
P,X Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 267, No. 22, issued 05 August 1992, W.C. Sessa et al., "Molecular Cloning and Expression of a cDNA Encoding Endothelial Cell Nitric Oxide Synthase", pages 15274-15276, entire document.	1, 4-8, 11, 13, 14, 22 2-3, 9-10, 15-21, 23-28
P,X Y	JOURNAL OF CLINICAL INVESTIGATION, Volume 90, issued November 1992, K. Nishida et al., "Molecular Cloning and Characterization of the Constitutive Bovine Aortic Endothelial Cell Nitric Oxide Synthase", pages 2092-2096, entire document.	1, 4-8, 11, 13 2-3, 9-10. 14-28
X Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, Volume 88, No. 23, issued 01 December 1991, J.S. Pollock et al., "Purification and Characterization of Particulate Endothelium Derived Relaxing Factor Synthase From Cultured and Native Bovine Aortic Endothelial Cells", pages 10480-10484, entire document.	8, 13, 14, 22, 25 1-7, 9-12, 15-21, 22-24, 26-28
Y	NATURE, Volume 351, issued 27 June 1991, D.S. Bredt et al., "Cloned and Expressed Nitric Oxide Synthase Structurally Resembles Cytochrome P-450 Reductase", pages 714-718, entire document.	1-28
Y	BRITISH JOURNAL OF PHARMACOLOGY, volume 92, No.3, issued November 1987, M.W. Radomski et al., "The Anti-aggregating Properties of Vascular Endothelium: Interactions Between Prostacyclin and Nitric Oxide", pages 639-646, especially pages 643-644.	23-24
Y	SEMINARS IN NEPHROLOGY, Volume 11, No. 2, issued March 1991, P.A. Marsden et al. "Nitric Oxide and Endothelins: Novel Autocrine/Paracrine Regulators of the Circulation", pages 169-185, especially page 172, first paragraph.	22
Y	MECHANISMS OF DISEASE, Volume 323, No. 1, issued 05 July 1990, J.R. Vane et al., "Regulatory Functions of the Vascular Endothelium", pages 27-36, especially page 31.	16-19, 25

International application No.
PCT/US93/01951

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12N 15/53, 15/63, 9/06, 9/88; C12Q 1/26; C12P 3/00; A61K 37/50, 48/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 172.3, 320.1, 252.3, 191, 168, 6, 232, 25; 536/23.2; 424/94.4; 514/44